Cardiovascular Research
doi:10.1093/cvr/cvx135

Amyloidogenic medin induces endothelial dysfunction and vascular inflammation through the receptor for advanced glycation endproducts

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Received 25 January 2017; revised 17 April 2017; editorial decision 21 June 2017; accepted 7 July 2017

Time for primary review: 35 days

Aims Medin is a common amyloidogenic protein in humans that accumulates in arteries with advanced age and has been implicated in vascular degeneration. Medin's effect on endothelial function remains unknown. The aims are to assess medin's effects on human arteriole endothelial function and identify potential mechanisms underlying medin-induced vascular injury.

Methods and results *Ex vivo* human adipose and leptomeningeal arterioles were exposed (1 h) to medin (0.1, 1, or 5 μM) without or with FPS–ZM1 [100 μM, receptor for advanced glycation endproducts (RAGE)-specific inhibitor] and endothelium-dependent function (acetylcholine dilator response) and endothelium-independent function (dilator response to nitric oxide donor diethylenetriamine NONOate) were compared with baseline control. Human umbilical vein endothelial cells were exposed to medin without or with FPS–ZM1 and oxidative and nitrative stress, cell viability, and pro-inflammatory signaling measures were obtained. Medin caused impaired endothelial function (vs. baseline response: -45.2 ± 5.1 and -35.8 ± 7.9% in adipose and leptomeningeal arterioles, respectively, each $P < 0.05$). Dilator response to NONOate was not significantly changed. Medin decreased arteriole and endothelial cell nitric oxide production, increased superoxide production, reduced endothelial cell viability, proliferation, and migration. Medin increased gene and protein expression of interleukin-6 and interleukin-8 via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Medin-induced endothelial dysfunction and oxidative stress were reversed by antioxidant polyethylene glycol superoxide dismutase and by RAGE inhibitor FPS-ZM1.

Conclusions Medin causes human microvascular endothelial dysfunction through oxidative and nitrative stress and promotes pro-inflammatory signaling in endothelial cells. These effects appear to be mediated via RAGE. The findings represent a potential novel mechanism of vascular injury.

Keywords Medin • Amyloid • Endothelial function • Inflammation • Oxidative stress

1. Introduction

Medin is an amyloidogenic peptide that accumulates with aging in various arterial beds, being found in all 18 patients studied that were 57 years or older[1] and reported to be present in 97% of a cohort of Caucasian subjects above 50 years of age.[2] Despite this ubiquity, little is known about the physiologic or pathologic effects of medin. Medin is a 50 amino acid peptide derived from milk fat globule protein epidermal growth factor 8 (MFG-E8).[1,3,4] It is amyloidogenic and forms vascular amyloid in the aorta and arteries of the upper part of the body, including intracranial vessels.[1] Prefibrillar medin was shown to induce aortic smooth muscle death *in vitro* while increasing the production of matrix–metalloproteinase-2,[3] suggesting a possible link between medin and aortic wall degeneration. We showed in a preliminary study that human endothelial cell cultures

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exposed acutely to medin demonstrated oxidative stress and reduced nitric oxide (·NO) bioavailability, although the underlying mechanism/s were not explored,[5] similar to effects observed with other amyloidogenic proteins (light chain proteins in AL amyloidosis[6] and β-amyloid[7]). The effects of medin on human microvascular endothelial function are not known. Our aim is to test the hypotheses that medin induces human arteriole endothelial dysfunction through oxidative/nitrative stress and reduced ·NO bioavailability, and that these effects are mediated via actions on receptor for advanced glycation endproducts (RAGE).

2. Methods

2.1 Medin peptide

Medin was expressed using pOPINS-medin in Lemo 21 (DE3) cells[8] and purified as previously described.[5] Expression was induced at OD600 ~1.0 with isopropyl-β-D-thiogalacto-pyranoside (1 mM) for 16 h at 20°C. Cells were harvested by centrifugation and pellets resuspended in 6 M guanidine hydrochloride (GdmCl), 20 mM sodium phosphate, 20 mM NaCl, pH 8.0. Cells were homogenized and cell debris removed by centrifugation. The supernatant was loaded onto a nickel-nitrilotriacetic acid (Ni^{2+}–NTA) column, washed with 6 M GdmCl, pH 8 and pH 6, and eluted with 6 M GdmCl, pH 2. Fusion protein was buffer exchanged into 20 mM Tris–Cl, 0.5 M NaCl, pH 7.4, and the His6–SUMO tag removed with SUMO protease I. The protease and the tag were removed using a Ni^{2+}–NTA column and the flow through containing medin buffer exchanged into phosphate buffered saline (PBS) and flash-frozen. Medin was confirmed at >95% purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis and characterized by matrix-assisted laser desorption and ionization mass spectrometry. Levels of endotoxin were assessed to be <0.5 ng/ml using the *Limulus* Amebocyte Lysate assay (Pierce, Dallas, TX).

2.2 Sources of human tissue

2.2.1 Aortic tissue

We assessed tissue concentration of medin from aortic tissue (10 ascending, 2 descending) from 12 human subjects (61.25 ± 3.98 years, range 39–81 years, four females, eight males). Tissue was obtained following informed consent from patients undergoing aortic surgery at Liverpool Heart and Chest Hospital through Liverpool Biobank, with approval granted by the Biobank Governance Board (Project number 15-06). The demographic information is provided in *Table 1*.

2.2.2 Adipose arterioles

Research volunteers scheduled to undergo elective clinically indicated abdominal surgery (inguinal or umbilical herniorrhaphy) and who were not known to have vascular disease or diabetes provided written, informed consent for subcutaneous adipose tissue donation ($N = 35$, 60.9 ± 2.3 years old, 1 female, 34 males by self-reported gender). The study was approved and under the supervision of the Phoenix Veterans Affairs Institutional Review Board (Migrino 001/004/1041).

2.2.3 Leptomeningeal arterioles

Leptomeningeal tissue were collected from cadavers following rapid autopsy (post-mortem interval 2.90 ± 0.2 h, median 2.87 h), who prior to death provided informed consent for brain donation post-mortem under the Brain and Body Donation program [www.brainandbodydonationprogram.org (18 July 2017)].[9] The donation program operations have been approved by the Banner Sun Health Research Institute Institutional Review Board. Tissues were immediately placed in sterile 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (4°C, pH 7.4). Demographic information on brain donors were as follows: $N = 13$, 88.1 ± 3.5 years old, four females, nine males. Neuropathologic diagnoses were as follows: Alzheimer's disease (AD) 4, vascular dementia 1, Parkinson's disease 4, mild cognitive impairment 2, and cognitively normal (age-consistent neuropathology only) 4. Additional details on demographic information on the brain donors are in Supplementary material online, *Table S1*.

All of the methods and procedures used conform to the principles outlined in the Declaration of Helsinki.

2.3 Determination of tissue medin content

Human aortic tissue was homogenized in PBS at 10% w/v (100 mg of wet tissue in 1 ml of PBS) using a MiniLys (Bertini Instruments, France). We performed a 40-times dilution of the patient samples (10% homogenate, followed by a 4-times sample dilution) to get the samples within the range of the standards 0–0.4 μM and then calculated the values back up to reflect the dilution process. Homogenized aorta samples and control medin at 0.1, 0.2, and 0.4 μM were mixed 1:1 with Novex tricine SDS sample buffer and heated to 70°C for 5 min. Samples were then loaded onto a 10–20% Novex tricine protein gel and subjected to electrophoresis in Novex tricine SDS running buffer according to the manufactures instructions (all Novex products sourced from Thermo Fisher Scientific, UK). Proteins were transferred to nitrocellulose (Protran 2 μm, Amersham GE Healthcare UK Ltd) for 1 h at 100 V. Western blots were blocked in 5% v/v donkey serum (EMD Millipore, UK) in PBS at room temperature with gentle shaking for 1 h. The blots were probed with medin antibody (custom antibody from GenicBio Hong Kong) at 1:1000 in PBS overnight at 4°C. Blots were thoroughly washed with PBS + 1% Tween 20 (PBST) and then incubated with a 1:10 000 dilution of the secondary antibody, horseradish peroxidase conjugated donkey anti-rabbit (GE Healthcare, UK) for 1 h at room temperature. The blots were washed again in PBST prior to chemiluminescent visualization using ELC (EMD Millipore, UK).

The blots were analyzed using the gel analyzer tools in ImageJ (National Institutes of Health, Bethesda, MD). A standard curve was plotted using the control medin samples and this was used to calculate the amount of medin in each of the tissue samples. No protein control for loading correction was assessed in the aortic tissue Western blot but investigators were careful in using a consistent %weight/volume amount of tissue for loading.

2.4 Human arteriole vasoreactivity

The details of the methodology have been previously reported.[6,7,10,11] Adipose arterioles (161 ± 10 μm lumen diameter) were isolated and following cannulation, were pressurized to 60 mmHg, the estimated physiologic pressure of similar sized arterioles *in vivo*. The vessels were preconstricted with increasing doses of endothelin-1 (10^{-9}–10^{-4} M) until 60% of maximum diameter was reached. Using videomicroscopy, baseline (control) dilator responses to acetylcholine (in successive doses of 10^{-9}–10^{-4} M) to assess endothelium-dependent vasodilation were measured. Separate vessels were measured for dilator response to 10^{-7}–10^{-4} M diethylenetriamine NONOate (DETA NONOate, Cayman Chemical, Ann Arbor, MI) to assess endothelium-independent vasodilation. This was followed by washout and then exposure (1 h) to medin 0.1, 1, and 5 μM (for adipose arterioles) and a second measurement of

Table 1 Demographic information on sources of aortic tissue

Participant	Age (years)	Gender	Aortic diameter (cm)	Medin tissue concentration (μM)	Cardiovascular risk factors/cardiac comorbidities
003	65	Male	5	12.12	Ex-smoker
004	48	Male	5.3	0	
005	81	Female	5	7.8	
006	48	Male	7	0	CAD, aortic valve replacement, hyperlipidemia, ex-smoker, stroke
007	51	Female	5.5	0	
014	67	Female	5.1	8.4	
015	65	Male	7	12	Aortic valve replacement, diabetes, hyperlipidemia, ex-smoker
016	77	Female	5.5	0	Heart failure, ex-smoker
017	77	Male	4.5	9.12	Ex-smoker
018	39	Male	5	0.92	
019	68	Male	N/A	13.72	
020	49	Male	5.5	0	Aortic valve replacement

CAD, coronary artery disease; N/A, data not available.

dilator responses to acetylcholine or DETA NONOate. Additional adipose arterioles were exposed to co-treatment of medin 5 μM, antioxidant polyethylene glycol superoxide dismutase (PEG-SOD, 300 U/ml, Sigma-Aldritch, St. Louis, MO), tetrahydrobiopterin (BH4, 100 μM, an essential cofactor for endothelial nitric oxide synthase [eNOS] function and maintenance of eNOS coupling,[12,13] Schircks Laboratories, Jona, Switzerland) or FPS-ZM1 (100 μM, a high-affinity RAGE-specific inhibitor,[14] Calbiochem, San Diego, CA). The maximum medin dose selected in this study (5 μM) is at par with physiologic/pathologic levels determined from our aortic tissue assays. Leptomeningeal arterioles underwent similar assessment of dilator response to acetylcholine at baseline, and after washout, post-treatment dilator responses were measured following 1 h exposure to 5 μM medin ± 100 μM FPS–ZM1 or 300 U/ml PEG-SOD.

2.5 Nitric oxide, peroxynitrite, and superoxide production and measurement of endothelial cell viability

The details of the methodology have previously been reported.[10] Frozen primary culture human umbilical vein endothelial cells (HUVECs, Lonza, Walkersville, MD) at passage 1 isolated from pooled donors by the corporate source were cultured and frozen down for future experiments as per the source's recommended protocols to form a working stock culture to draw from. Fresh cultures were seeded from these stocks and cells (passages 5–8) were allowed to grow to full confluence before treatment. Individual experimental treatments were in separate seeded cultures from the frozen stocks. HUVECs seeded into 10 cm^2 conical culture tubes each with a glass cover slip at the bottom were treated with vehicle control, medin 5 μM without or with PEG-SOD (300 U/ml), FPS–ZM1 (100 μM), or BH4 (100 μM) for 1 h and medin 5 μM without or with FPS–ZM1 (100 μM) for 20 h.

·NO, superoxide, and peroxynitrite production were measured in endothelial cells exposed to treatment for 1 and 20 h while endothelial cell viability was measured following exposure to treatment for 20 h. ·NO production was assessed by directly measuring ·NO head gas production for cells treated for 20 h, and using 4,5-diaminofluorescein diacetate (DAF-2 DA) fluorescence for cells and arterioles treated for 1 h, a well-validated fluorescence method to indirectly measure ·NO production.[15] Head gas was measured by piercing the seal of the conical tube and using Sievers 280 nitric oxide Analyzer (General Electric Analytical Instruments, Waukesha, WI) and normalized to cell count. For fluorescence microscopy measurements, endothelial cell-containing glass coverslips were removed from the culture tubes, transferred into 12-well plates, washed, and then stained for 15 min with 5 μmol/l dihydroethidium (Thermo Fisher Scientific, Waltham, MA), a marker of superoxide production[16] and 20 μmol/l coumarin boronate pinacolate ester (Cayman Chemical, Ann Arbor, MI), a marker of peroxynitrite production[17] in HEPES buffer containing 10^{-4} M acetylcholine. ·NO was assessed in HUVECs treated for 1 h by exposing cells to treatment (vehicle, medin ± PEG-SOD or BH4 or FPS–ZM1) for 1 h, administration of acetylcholine (10^{-4} M) and staining with DAF-2 DA (10 μM, Life Technologies, Carlsbad, CA) 45 min into treatment exposure allowing 15 minutes of staining. Cells were then washed and fixed in 4% formaldehyde in PBS followed by cold methanol and mounted on glass slides for imaging. Using EVOS FL Auto (Life Technologies), reacted dihydroethidium products were imaged using the RFP light cube (excitation 531/40 nm; emission 593/40 nm), reacted coumarin boronate were imaged using the DAPI light cube (excitation 357/44 nm; emission 447/60 nm) and reacted DAF-2 DA were imaged using the GFP light cube (excitation 470/22 nm; emission 510/42 nm). Cell fluorescent signals were measured using ImageJ 1.49 analysis software and results expressed as values relative to control. For 20-h treated cells, the remaining cells from the culture tubes were lifted with trypsin into flow cytometry tubes, washed, and resuspended in 10 nM calcein acetyoxymethyl (Life Technologies) HEPES buffer containing 1.6 mM calcium for 15 min and then washed. This non-fluorescent compound is a marker of cell viability as presence of intact cellular endogenous esterases will hydrolyze the compound and release fluorescent calcein anion.[18] Cellular fluorescent signals were measured using Beckman-Coulter FC500 flow cytometer (Indianapolis, IN) reading with excitation at 488 nm on the FL-1 channel. Results are expressed as values relative to control. In separate experiments, HUVECs were treated for 20 h with vehicle, medin 5 μM without or with $\alpha,\beta,\gamma,\delta$-tetrakis(4-*N*-methylpyridyl) porphine (FeTMPyP, 50, 100, and 200 μM), a peroxynitrite decomposition catalyst,[19] and cell viability was assessed using calcein acetyoxymethyl fluorescence as described.

Adipose arterioles were exposed to 1 h treatment with vehicle control, medin 5 μM ± PEG–SOD (300 U/ml) or FPS–ZM1 (100 μM) and ·NO was measured using DAF-2 DA staining while superoxide was measured using dihydroethidium staining similar to methods described above.

2.6 Endothelial cell proliferation and migration assay

To assess the effect of medin on cellular proliferation, HUVECs were plated in a 96-well plate at 8000 cells per well. After 24 h, cells were treated with medin 5 μM or vehicle and proliferation assay was performed on cells treated on day 0 and 2 days following treatment using Alamar Blue Assay (Thermo Fisher Scientific) modified from established protocol.[20] To perform the proliferation assay, media was removed from the well plate and the samples were washed three times with DPBS. The Alamar Blue solution was prepared in warmed culture media at concentration of 10% (v/v) and then added to each well followed by incubation for 4 h. Afterwards, fluorescence intensity was measured using a fluorescent plate reader (FLUOstar Omega, BMG LabTech, Ortenberg, Germany) at 544–590 nm wavelength.

A scratch-wound assay, modified from established protocol,[21] was utilized to analyze the effect of medin on endothelial cell migration. HUVECs were plated in 24-well plate and incubated until 90% confluent. Later, a pipette tip (20 μl) was used to perform a straight scratch in the well. The wells were next washed with DPBS and replaced with cell culture media as a control or media containing medin 5 μM. The 24 well-plate was placed into a microscope incubator and imaged for 8 h overnight. Next, each time point was analyzed by making 10 distance measurements between the scratch. Distance migrated were quantified in ImageJ 1.49 analysis software.

2.7 Pro-inflammatory signaling assays

The methodology for detecting gene and protein expression has been previously reported.[10] Quantitative polymerase chain reaction was used to measure gene expression. HUVECs (passages 4–8) in LONZA EGM-2 endothelial cell growth media were exposed to 20 h of vehicle control, medin 1 or 5 μM, without or with co-treatment of either FPS-ZM1 (100 μM) or RO106-9920 (1, 10, or 100 μM, Tocris Biosciences, Bristol, UK), a small molecule inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-dependent cytokine expression.[22] After cell lysis, RNA was extracted and converted to cDNA using Aurum Total RNA Mini Kit and iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Primers for inflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) were purchased from Integrated DNA Technologies and polymerization signal was measured using SybrGreen on the Thermal Cycler CFX96 Real-Time System (Bio-Rad Laboratories, Coralville, IA) with β-actin serving as reference gene for normalization. In separate experiments, HUVECs were treated for 20 h with vehicle control, medin 5 μM without or with either PEG-SOD (300 U/ml) or FeTMPyP (200 μM) and IL-6 and IL-8 gene expression were measured using similar methods.

The conditioned cell media from above-treated HUVECs were collected for measurement of IL-6 and IL-8 protein and stored at -80°C until time of enzyme linked immunosorbent assay (ELISA). Conditioned media samples were thawed on ice and assayed either diluted 1:10 for IL-8 or assayed whole for IL-6 ELISA to keep values within range of the kit standards. Samples were assayed in duplicate as per instructions of the appropriate Human IL-6 or Human IL-8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

2.8 Western blot assay

We measured total eNOS, phosphorylated eNOS (both serine 1177 and threonine 495) and computed phosphorylated eNOS:total eNOS ratio in HUVECs treated for 1 h with vehicle control and medin 5 μM± FPS–ZM1 (100 μM). In separate cells, we assessed whether medin's pro-inflammatory effect is through NFκB-mediated cytokine production, HUVECs were exposed for 5 h to vehicle control, medin 1 or 5 μM without or with FPS–ZM1 (100 μM). Cells were lysed in complete radioimmunoprecipitation assay-based lysis buffer with phosphatase inhibitor cocktail 2 and protease inhibitor (Sigma-Aldritch). 30 μg of protein (determined by Bradford assay) was loaded for electrophoresis in precast MiniProtean TGX 4–20% gel (Bio-Rad Laboratories). Proteins were transferred to polyvinylidene fluoride low fluorescence membrane for western blot at 100 V for 50 min. Antibodies against total eNOS (Sigma-Aldritch), phosphorylated eNOS (Cell Signaling, Danver, MA), phosphorylated NFκB (Cell Signaling) at 1:500, 1:1000, and 1:1000 dilution, respectively, were used and all membranes blocked a second time before developing via 680 RD or 800 CW infrared fluorescent conjugated goat secondary antibody (Li-COR Biosciences, Lincoln, NE) 1 to 15 000 dilution. Fluorescent bands in the infrared range were detected using Li-COR Odyssey CLx imaging system. Signal was measured using Image Studio 4.0 (Li-COR) normalized to β-actin and results are expressed as values relative to control.

2.9 Data and statistical analyses

Data are expressed as mean ± standard error of means with significant *P*-value (two-sided) set at $P < 0.05$. Arteriole dilator responses (response to acetylcholine or DETA-NONOate) were assessed by comparing post-treatment response with baseline control response using 2-way repeated measures analysis of variance (ANOVA) with pairwise post-hoc analysis using Holm-Sidak method, while comparison between two treatment responses were analyzed using 2-way ANOVA with pairwise post-hoc analysis using Holm-Sidak method. Acetylcholine half-maximal effective concentration (EC50, estimated dose that produces dilation to 50% of maximum dilation) was derived using nonlinear regression with a variable slope (four parameters) and least squares ordinary fit (GraphPad Prism 5.0, GraphPad software, La Jolla, CA) similar to our previous methods[6,10] and compared between baseline and treatment response using paired Student *t*-test. For endothelial cell experiments, each data point (N) is a replicate that represents a separate independent HUVEC batch. For non-normally distributed data, natural log (Ln) transformation was first performed and ANOVA performed using normally distributed Ln transformed data. For endothelial cell assays, group treatment analyses were assessed using one-way repeated-measures analysis of variance (ANOVA) with post-hoc testing using Holm–Sidak pairwise comparison for normally distributed data. For datasets with treatment sample sizes that are not equal and data that are not normally distributed, one way ANOVA on ranks was done and Dunn's post hoc pairwise comparison testing performed. Paired comparisons that are not normally distributed were analyzed using Wilcoxon signed rank test. Statistical analyses were performed using Sigmastat 3.5 (Systat, San Jose, CA).

3. Results

Monomeric medin tissue concentration was determined from the aortic tissue of 12 donor participants. The range was 0–75 μg/g wet tissue (29.1 ± 8.9 μg/g). Assuming an aortic tissue density of 1 g/ml,[23] this corresponds to 0–13.72 μM (mean 5.34, standard deviation 5.66,

median 4.36, interquartile range 4.36–9.84 μM) (*Figure 1A* and *B*). This physiologic dose range was used for our arteriole and endothelial cell experiments. There was significant correlation between aortic tissue medin content and age ($R = 0.592$, $P = 0.04$). Subjects who were 55 years old or older had significantly higher aortic medin than those <55 years old.

Medin dose-dependently impaired peripheral adipose arteriole dilator response to acetylcholine signifying impairment of endothelial function (change vs. baseline control, medin 0.1, 1, and 5 μM: +4.2 ± 3.1, -23.1 ± 5.3, -45.2 ± 5.1%, respectively, *Figure 2A–C*). Acetylcholine LogM EC50 was significantly higher (less negative) for arterioles treated with medin 1 μM (-5.87 ± 0.44 vs. -6.88 ± 0.17 control, $P = 0.03$) and 5 μM (-4.6 ± 0.32 vs. -7.07 ± 0.21 control, $P < 0.001$), but not 0.1 μM (control -7.4 ± 0.69, medin 0.1 μM -7.82 ± 0.42). There was no significant reduction in dilator response to increasing doses of DETA NONOate, signifying lack of effect on endothelium-independent function (*Figure 2D*). DETA NONOate LogM EC50 for medin 5 μM (-5.76 ± 0.31) was not significantly different vs. control (-5.78 ± 0.0.28). The results were similar in central leptomeningeal arterioles with medin-induced reduction in dilator response to acetylcholine (-34.8 ± 6.5% vs. control) (*Figure 2E*). Acetylcholine LogM EC50 was significantly higher for leptomeningeal arterioles treated with medin 5 μM vs. controls (-4.9 ± 0.8 vs. -6.7 ± 0.8, $P < 0.001$).

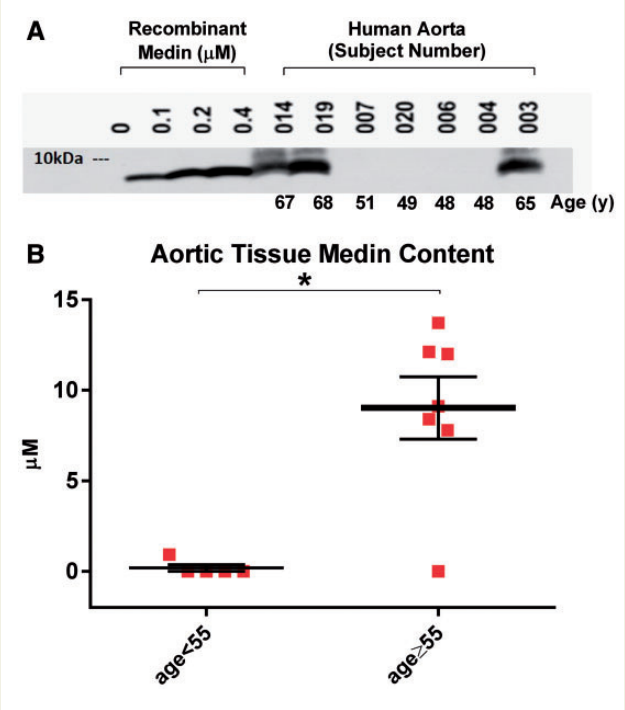

Figure 1 Aortic tissue medin. (*A*) Western blot using anti-human medin antibody from varying doses of recombinant medin (*left side of panel*) and from representative donor human aortic tissues (*right side of panel*). This shows variable protein expression of monomeric medin among various subjects with subjects' ages shown at the bottom of the figure. Note that aortic tissue loading was diluted to get the samples within the range of the standards as detailed in Methods section. (*B*) Comparison of aortic tissue medin concentration by age show increased medin content in the aorta of subjects ≥55 years old vs. younger, suggesting age-related differences in medin accumulation in aortic tissue ($N = 5$, age <55, $N = 7$, age ≥55). *$P < 0.05$.

Adipose arterioles exposed for 1 h to medin 5 μM showed reduced ·NO and increased superoxide and peroxynitrite (*Figure 2F–H*). Similar results were seen in HUVECs for ·NO and superoxide, however, the difference in peroxynitrite production between control and medin-treated cells did not reach statistical significance following 1 h exposure, unlike results following 20-h exposure (see results below).

To assess the role of oxidative stress in causing endothelial dysfunction, adipose and leptomeningeal arterioles were co-treated with antioxidant PEG–SOD and medin. PEG–SOD restored arteriole dilator response to acetylcholine (*Figure 3A–D*). PEG-SOD also restored ·NO production and reduced superoxide production in endothelial cells treated with medin (*Figure 3E–F*).

To assess whether medin's effect on reducing endothelial cell ·NO and increasing superoxide is related to eNOS uncoupling, we measured phosphorylated and total eNOS. Endothelial cells treated with medin for 1 h did not show any difference with control in phosphorylated eNOS (serine 177 and threonine 495), total eNOS and phosphorylated eNOS/total eNOS ratios (Supplementary material online, *Figure S1A–H*). Adipose arterioles exposed to 1 h of medin plus essential NO synthase co-factor BH4 restored dilator response to acetylcholine compared with exposure to medin alone (Supplementary material online, *Figure S1I*). In addition, co-treatment for 1 h with medin and BH4 restored NO and reduced superoxide production in endothelial cells when compared to medin treatment alone (Supplementary material online, *Figure S1J-K*); no group differences were noted in peroxynitrite production following 1 h treatment (Supplementary material online, *Figure S1L*).

In order to assess the role of RAGE in mediating the vascular effects of medin, adipose arterioles were exposed to medin co-treated with FPS–ZM1 (100 μM), a multimodal, high affinity and specific inhibitor of RAGE.[14] Arteriole dilator response was compared between arterioles treated with medin and with medin co-treated with RAGE inhibitor FPS–ZM1. FPS–ZM1 restored dilator response to acetylcholine signifying preservation of endothelial function in both adipose and leptomeningeal arterioles (*Figure 4A–D*). There was a trend toward restoration of NO production with FPS–ZM1 co-treatment (*Figure 4E*) that did not reach statistical significance on pre-specified pairwise post-hoc analysis following repeated measures one way analysis of variance. However, paired *t*-test analysis of arteriole NO production of medin vs. medin + FPS–ZM1 treatment showed significant difference (relative to control: 0.56 ± 0.9 vs. 0.89 ± 0.19 respectively, $N = 7$, $P = 0.04$). Furthermore, HUVECs treated for 1 h with medin + FPS–ZM1 showed significantly higher ·NO compared with medin-treated alone (signal relative to vehicle-control: 1.0 ± 0, medin 5 μM: 0.63 ± 0.05, medin + FPS–ZM1 100 μM: 0.94 ± 0.14, $P < 0.05$ medin vs. C, $P < 0.05$ medin vs. medin + FPS–ZM1, $n = 10$), similar to results obtained with 20 h exposure (see results below). FPS–ZM1 co-treatment resulted in reduced superoxide and peroxynitrite production in adipose arterioles following 1-h exposure (*Figure 4F* and *G*). FPS–ZM1 co-treatment or FPS-ZM1 alone did not change levels of phosphorylated eNOS (both serine 177 and threonine 495) or phosphorylated eNOS/total eNOS ratio (Supplementary material online, *Figure S2A–H*).

We also assessed the effects of medin on endothelial cell viability and oxidative stress following 20-h exposure. There was dose-dependent reduction in ·NO and increase in peroxynitrite (*Figure 5A* and *C*); medin also increased superoxide production and reduced cell viability (*Figure 5B* and *D*). Co-treatment of medin with peroxynitrite decomposition

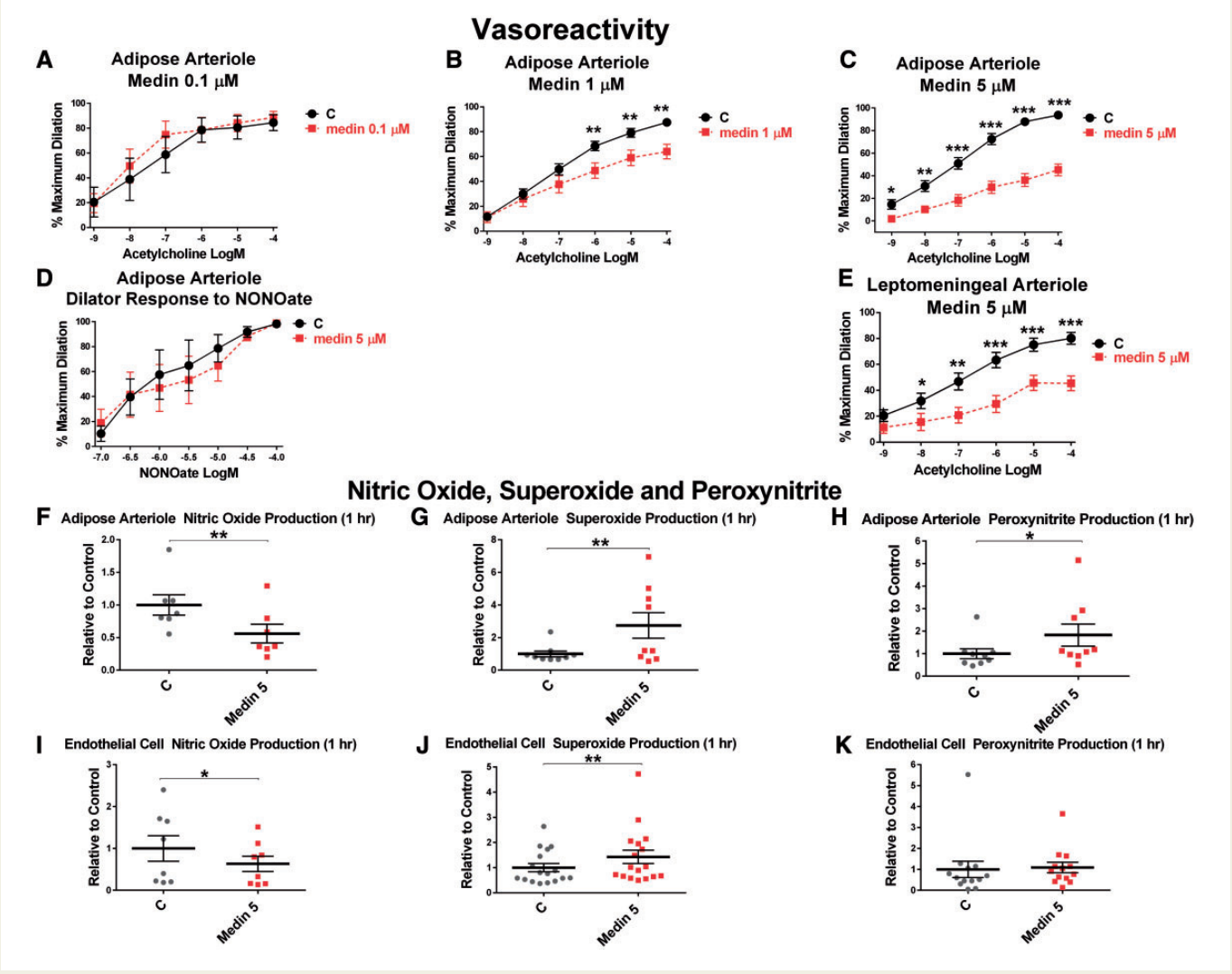

Figure 2 Effects of medin on human arteriole vasoreactivity and oxidative and nitrative stress. (*A–C*) Shows dilator response to acetylcholine in human adipose arterioles exposed to increasing doses of medin for 1 h. There was dose-dependent reduction in dilator response to acetylcholine in medin-treated arterioles signifying endothelium-dependent vasodilation. (*D*) Shows dilator response to ·NO donor DETA–NONOate in adipose arterioles exposed to medin 5 μM for 1 h. There was no significant change in dilator response suggesting medin does not impair endothelium-independent vasodilation. (*E*) Shows that similar to response in adipose arterioles, medin-treated leptomeningeal arterioles show endothelial dysfunction with impaired dilator response to acetylcholine. To assess the bases of observed endothelial function in human arterioles treated with medin, NO, superoxide, and peroxynitrite levels were measured in adipose arterioles (*F–H*) and endothelial cells (*I–K*) treated with medin for 1 h. In both adipose arterioles and endothelial cell cultures, there was reduction in NO and increase in superoxide production signifying medin-induced oxidative stress and reduced ·NO bioavailability. There was significant reduction in peroxynitrite in adipose arterioles but not in endothelial cells with 1 h treatment. *A N* = 3, *B N* = 14, *C N* = 23, *D N* = 5, *E N* = 13, *F N* = 7 each, *G N* = 9 each, *H*: *N* = 9 each, *I*: *N* = 8 each, *J*: *N* = 17 each, *K N* = 13 each; *A–E* 2-way repeated measures ANOVA with post-hoc pairwise Holm–Sidak test, *F–K* paired *t*-test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 control vs. treatment group.

catalyst FeTMPyP (200 μM) restored endothelial cell viability (*Figure 5E*). Medin also caused reduced endothelial cell proliferation and reduction in endothelial cell migration speed (*Figure 5F* and *G*).

Medin exposure for 20 h also caused increased gene and protein expression of IL-6 and IL-8 in HUVECs (*Figure 6A–D*). There was a dose-dependent increase in endothelial cell phospho–NFκB following 5 h of exposure to medin (*Figure 6E* and *F*). Additional replicates were performed to confirm the effects of medin on gene expression of IL-6 and IL-8, and results were consistent in showing significant increase in IL-6 (relative to control: control 1 ± 0, medin 5 μM 22.8 ± 4.7, *N* = 33, *P* < 0.001) and IL-8 (relative to control: control 1 ± 0, medin 5 μM 6519.4 ± 2413, *N* = 34, *P* < 0.001) (paired Wilcoxon signed rank test). The increase in IL-6 and IL-8 induced by medin is likely related to increased activation of NFκB as co-treatment with RO106-9920, an inhibitor of NFκB-dependent cytokine expression,[22] reversed medin's effect on stimulating gene and protein expression of IL-6 and IL-8 (*Figure 6G–J*). Co-treatment of medin with either PEG-SOD or peroxynitrite catalyst FeTMPyP did not reverse the increased IL-6 and IL-8 gene expressions induced by medin in HUVECs (Supplementary material online, *Figure S3*).

Co-treatment of medin with FPS-ZM1 also restored 20 h endothelial cell ·NO production and cell viability while reversing medin-induced

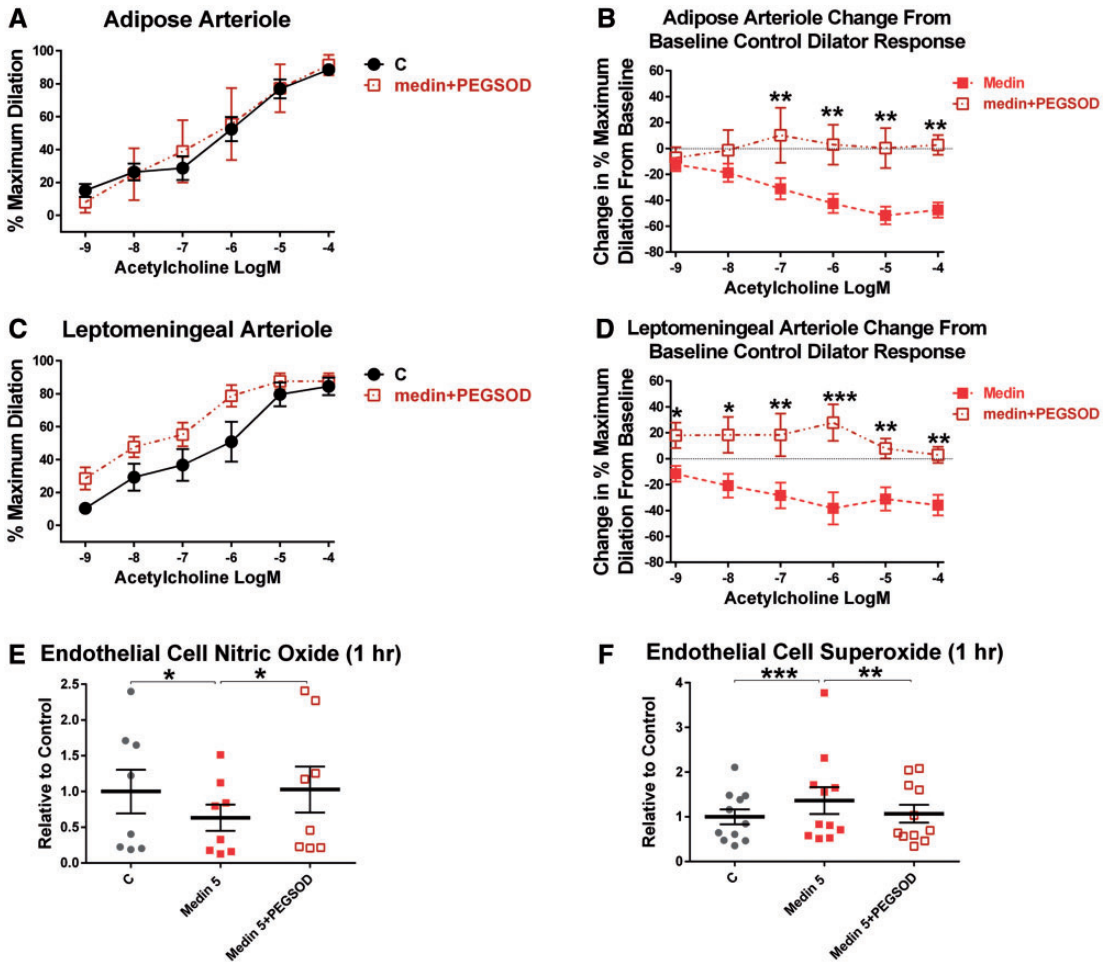

Figure 3 Restoration of medin-induced endothelial dysfunction by antioxidant PEG–SOD. (*A, C*) Show no significant change in dilator response compared with control in adipose and leptomeningeal arterioles treated with medin and antioxidant polyethylene glycol superoxide dismutase (PEG–SOD, 300 U/ml), in contrast to *Figure 2C and* E. (*B, D*) Represent the difference in dilator response to acetylcholine following treatment and dilator response to acetylcholine at baseline (control). These show that medin treatment (5 μM) resulted in reduction of dilator response to acetylcholine in both adipose and leptomeningeal arterioles that was not seen when medin was co-treated PEG–SOD, signifying restoration of endothelium-dependent vasodilation by PEG–SOD. (*E–F*) Shows that co-treatment of medin 5 μM with PEG–SOD (300 U/ml) for 1 h restores ·NO and reduces superoxide production. *A:N* = 5 each, *B*: medin *N* = 20, medin + PEG–SOD *N* = 5, *C*: *N* = 5 each, *D*: medin *N* = 13, medin + PEGSOD *N* = 5, *E N* = 8 each and *F N* = 11 each. *A, C* were analyzed using 2-way repeated measures ANOVA; *B, D* were analyzed using 2-way ANOVA with post-hoc Holm–Sidak pairwise test; *E, F* were analyzed using one-way repeated ANOVA with post-hoc Holm–Sidak pairwise test; **P* < 0.05, ***P* < 0.01, ****P* < 0.01 (in *B, D* control vs. treated group, in *E, F* groups in bracket).

increase in peroxynitrite production (*Figure 7A–C*). FPS–ZM1 co-treatment also prevented phosphorylation of NFκB by medin as well as the increase in gene and protein expression of IL-6 and IL-8 (*Figure 7D–I*).

4. Discussion

The study presents the following novel findings. First, medin induced human arteriole and endothelial cell dysfunction likely through increased oxidative and nitrative stress. Second, medin caused profound pro-inflammatory signaling effect on endothelial cells mediated through activation of NFκB. Third, these adverse effects appear to be mediated via RAGE. These findings demonstrate a new mechanism of injury on human microvessels induced by medin that could be relevant in aging-related vascular pathology.

4.1 Endothelial dysfunction induced by medin

Despite reports suggesting that medin is a ubiquitous human amyloidogenic protein, being present in almost 100% of Caucasian subjects studied over 50 years of age,[2,24] little is known about medin's pathophysiologic effects. Consistent with previous reports demonstrating increased arterial medin content with advanced age,[1–4] we also show significant positive correlation between age and medin content in aortic tissue, with significantly increased medin in subjects ≥55 years old vs. younger. Because previously published reports[1–4] on medin content in human vessels plus our current study still represent <100 total subjects studied, it is imperative that the epidemiology of medin vascular deposition be studied in larger subsets of patients to confirm findings of high prevalence with aging in a large population.

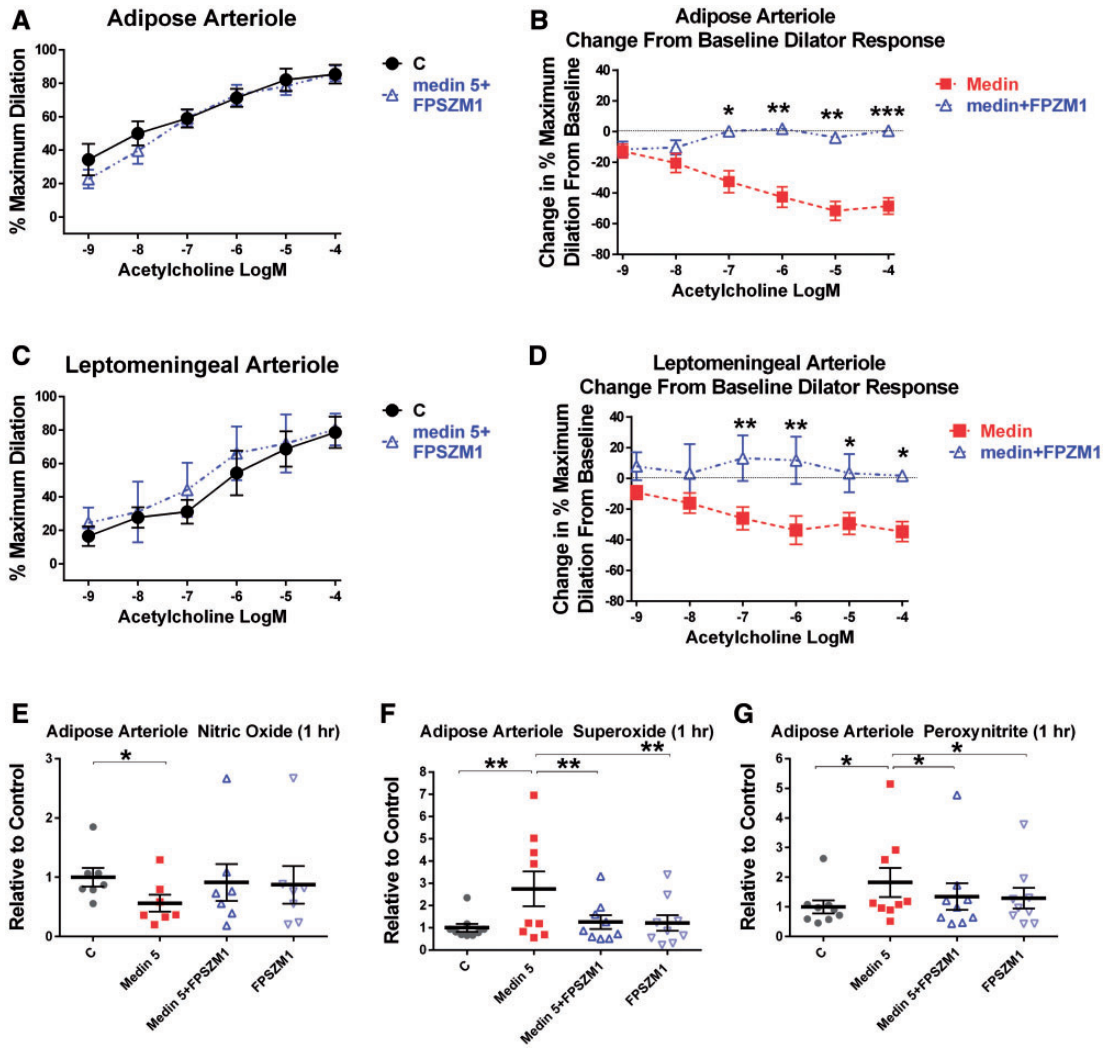

Figure 4 Restoration of medin-induced endothelial dysfunction by RAGE inhibitor. Co-treatment with medin and RAGE inhibitor FPS–ZM1 (100 μM) in adipose (*A*) or leptomeningeal (*C*) arterioles did not result in impaired dilation to acetylcholine compared with control. The change in dilator response from baseline control was significantly different between vessels treated with medin vs. medin + FPS–ZM1 (*B, D*) showing that FPS–ZM1 restored dilator response of arterioles exposed to medin. (*E*) Co-treatment with FPS–ZM1 showed a trend, but not a statistically significant difference in ·NO production in adipose arterioles treated with medin by pairwise post-hoc analysis following one way repeat measures ANOVA. However, paired *t*-test of ·NO production of medin vs. medin + FPS–ZM1 treatment showed significant difference ($P = 0.04$). (*F, G*) Shows that co-treatment of FPS–ZM1 with medin reduced adipose arteriole superoxide and peroxynitrite production. *A* medin $N = 23$, medin + FPS–ZM1; $N = 5$, *B*: medin $N = 13$, medin + FPS–ZM1 $N = 5$, *C*: $N = 7$ each, *D, E*: $N = 9$ each; *A, C*: 2-way repeated measures ANOVA with post-hoc pairwise Holm-Sidak test; *B, D*: 2-way ANOVA with post-hoc pairwise Holm–Sidak test; *E-G*: 1-way repeated measures ANOVA with post-hoc pairwise Holm–Sidak test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (in *B, D* control vs. treated group, in *E–G* groups in bracket).

Our results show that medin induces profound endothelial dysfunction in human peripheral adipose and central leptomeningeal arterioles while reducing endothelial cell viability, migration and proliferation. This is likely due to induction of oxidative and nitrative stress (increased superoxide and peroxynitrite) leading to decreased ·NO bioavailability, because co-treatment with superoxide scavenger PEG–SOD restored endothelial function, reduced superoxide, and increased ·NO production. One possible mechanism underlying medin's oxidative stress effects may be due to eNOS uncoupling since our data showed no change in phospho–eNOS/eNOS ratio with medin treatment (suggesting diversion of eNOS activity from NO to superoxide production) and restoration of endothelial function, NO production with reduced superoxide following co-treatment with BH4, a nitric oxide synthase co-factor that promotes eNOS coupling. Because endothelial dysfunction is an early and key abnormality that underlies aging-related arterial diseases including essential hypertension and atherosclerosis,[25] our observations suggest a potential novel mechanism of vascular injury. Our results show that medin does not affect arteriole dilator response to DETA–NONOate, an NO donor, suggesting that medin does not affect downstream signaling of NO through guanylate cyclase and cyclic guanosine monophosphate (cGMP) to produce vasodilation.

Our results show reduced endothelial cell viability following exposure to medin in the setting of increased superoxide and peroxynitrite production. NO and superoxide react and form as primary intermediate

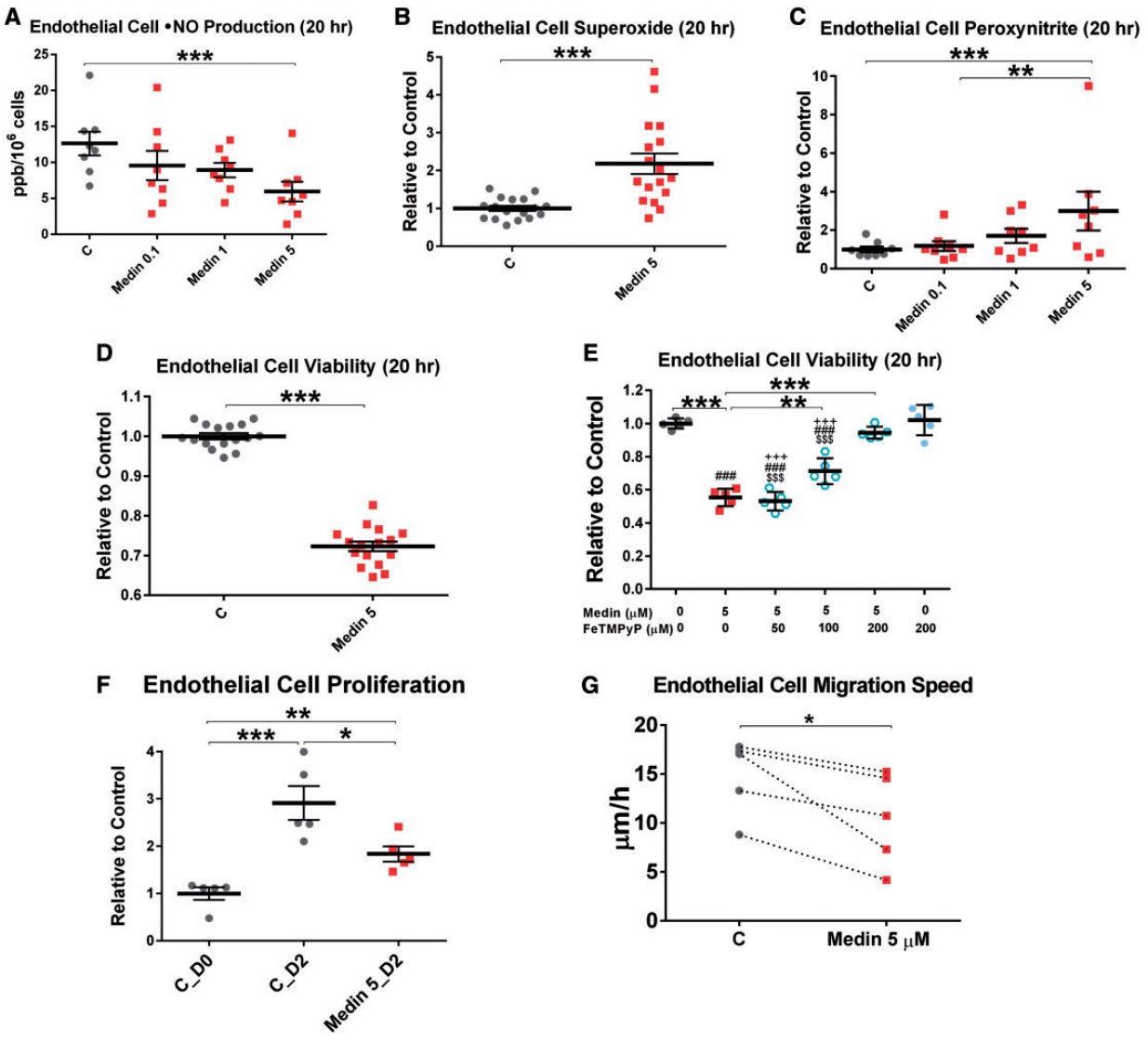

Figure 5 Adverse effects of medin on endothelial cell viability and function. In *A–E*, HUVECs were treated with medin for 20 h. There was reduction in NO, increase in superoxide and peroxynitrite production (*A–C*). There was reduced cell viability (*D*). In separate independent experiments, co-treatment with peroxynitrite catalyst FeTMPyP restored viability in cells treated with medin (*E*). These suggest that medin's adverse effect on endothelial cell viability is mediated by oxidative/nitrative stress. Medin also caused reduction in endothelial cell proliferation over 2 days (*F*) and reduced endothelial cell migration in 24 h following scratch wound assay (*G*). Note that in *G*, connecting lines represent data from paired independent experiments to facilitate data representation (cells were separately treated with either vehicle or medin). *A*: $N = 8$ each, *B*: $N = 23$ each, *C*: $N = 8$ each, *D*: $N = 16$ each, *E–G*: $N = 5$ each; *A, C, E and F* were analyzed using repeated measures ANOVA with post-hoc pairwise Holm–Sidak, *B, D and G* were analyzed using paired *t*-test; $^{*}P < 0.05$, $^{***}P < 0.001$, $^{+++}P < 0.001$ vs. FeTMPyP 200 μM, $^{\#\#\#}P < 0.001$ vs. control, $^{\$\$\$}P < 0.001$ vs. medin 5 μM + FeTMPyP 200 μM.

the potent oxidant peroxynitrite.[17] Peroxynitrite is a potent mediator of cellular damage in a wide range of conditions including cardiovascular disease.[26] Our results demonstrated that FeTMPyP, a peroxynitrite decomposition catalyst, restored endothelial cell viability in the setting of medin exposure, suggesting that medin's effect on cell viability is mediated via peroxynitrite.

Using a high-affinity specific inhibitor, we demonstrated that the effects of medin are mediated via RAGE. Another amyloidogenic peptide, β-amyloid, uses the same receptor to mediate perturbations in cerebral vessels, neurons and microglia in AD.[27] Indeed, we previously showed that similar to medin, Aβ42 induces endothelial dysfunction in leptomeningeal and adipose arterioles through reduced ·NO bioavailability and induction of oxidative and nitrative stress.[7,11] Since both medin and Aβ were shown to be present in cerebral vessels especially with advanced age, it is important in future studies to fully define contributing effects on vascular function and possible interactions by both amyloid proteins.

4.2 Medin and proinflammatory vascular signaling

Our findings show that medin also induces pro-inflammatory signaling in endothelial cells with increased gene and protein expression of IL-6

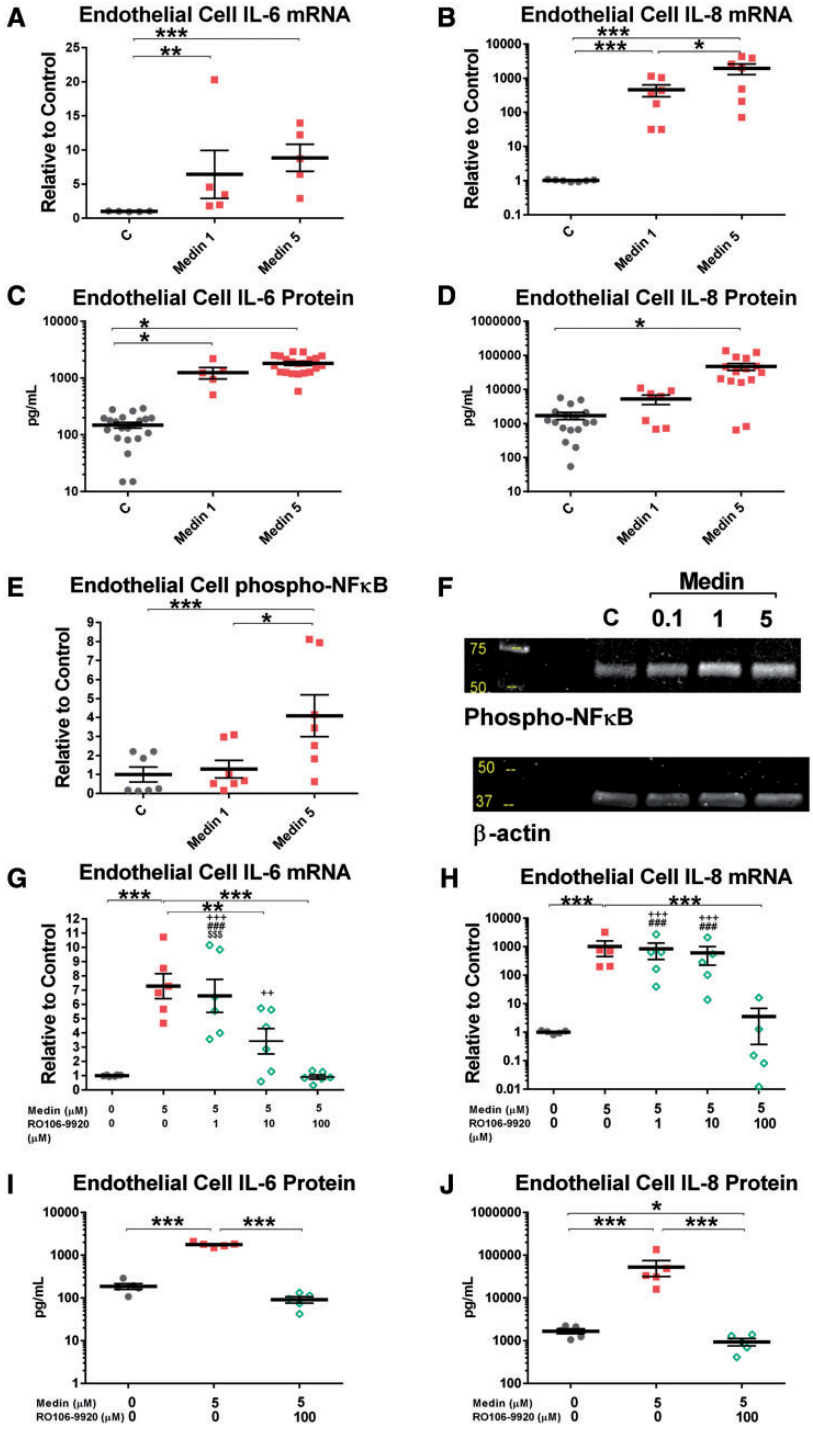

Figure 6 Medin induces pro-inflammatory cytokine production in endothelial cells. In *A–D*, endothelial cells were exposed for 20 h to increasing doses of medin. There was increased gene expression of interleukin (IL)-6 and IL-8 following exposure to medin 1 and 5 μM and corresponding increase in IL-6 and IL-8 protein expression with medin 5 μM dose showing that medin induces pro-inflammatory signaling in endothelial cells. *E–F* Shows that medin increased levels of phosphorylated NFκB, a protein complex known to control transcription of cytokine production. Co-treatment with NFκB inhibitor RO106-9920 prevented medin-induced increases in IL-6 and IL-8 gene (*G, H*) and protein (*I, J*) expression, suggesting that the proinflammatory effects of medin is through activation of NFκB. Note that to facilitate data representation, logarithmic scale was used in *A-D* and *H*. *A*: $N = 5$ each, *B:* $N = 7$ each, *C*: *C*—$N = 21$, medin 1—$N = 5$, medin 5—$N = 21$; *D*: *C*—$N = 17$, medin 1—$N = 7$, medin 5—$N = 15$, *E*: C—$N = 11$, medin 0.1—$N = 3$, medin 1—$N = 5$, medin 5—$N = 11$; *A–B, E, G–J* were analyzed using repeated measures ANOVA with post-hoc pairwise Holm-Sidak, *C–D* were analyzed using one-way ANOVA with post-hoc pairwise analyses by Dunn's method; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

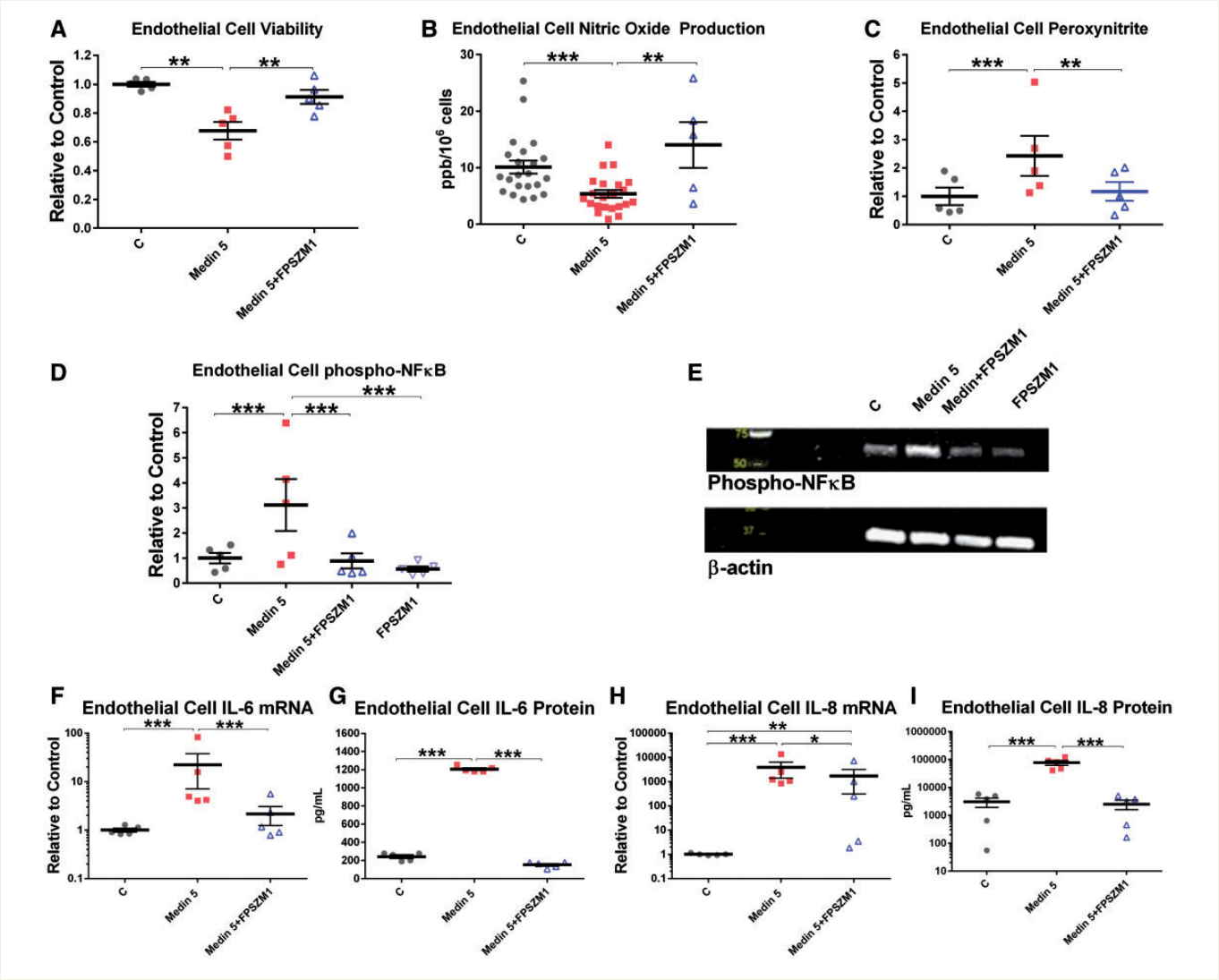

Figure 7 RAGE inhibitor protects viability of endothelial cells and prevents pro-inflammatory signaling following medin treatment. In *A–C*, co-treatment for 20 h of RAGE inhibitor FPS-ZM1 (100 μM) with medin restored endothelial cell viability, restored NO production and reduced peroxynitrite production compared with medin treatment alone. *D–E* Shows that FPS–ZM1 co-treatment with medin also reduces phosphorylated NFκB levels, gene and protein expression of interleukin (IL)-6 and IL-8. Note that to facilitate data representation, logarithmic scale was used in *F*, *H–I*. *A*, *C-D* and *F–I*: $N = 5$ each, *B*: *C*—$N = 22$, medin—$N = 22$, medin + FPS–ZM1—$N = 5$; *A*, *C*, *D*, *F–I* were analyzed using repeated measures ANOVA with post-hoc pairwise Holm–Sidak, *B* was analyzed using 1-way ANOVA with post-hoc pairwise Holm–Sidak; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and IL-8. This is likely mediated through NFκB signaling as supported by the increase in phospho–NFκB and the reversal of pro-inflammatory signaling by NFκB inhibitor RO106-9920. Interleukin-6 and IL-8 function as chemoattractants to neutrophils, basophils, and lymphocytes[28] and are also upregulated in the presence of other cardiovascular risk factors such as hyperlipidemia, smoking and diabetes.[29] In addition, studies suggest that IL-8 may also promote angiogenesis.[30] A potential implication is that the presence of medin could possibly amplify pro-inflammatory and oxidative stress signaling induced by other risk factors. Medin's stimulatory effects on gene expression of IL-6 and IL-8 were not reversed by co-treatment with PEG–SOD or FeTMPyp at doses that reversed medin-induced reduction of endothelial cell viability, suggesting that medin's pro-inflammatory effect is independent of superoxide or peroxynitrite generation. Although peroxynitrite has been shown to activate NFκB-dependent proinflammatory signaling in human polymorphonuclear cells[26,31,32] and monocytes,[33] other investigators showed the opposite effect in other types of cells,[26,34] including endothelial cells,[35] suggesting that the relationship between peroxynitrite and NFκB signaling is complex and may be cell-type and condition dependent.

The effects on immune signaling with chronic exposure remains to be studied, but if the pro-inflammatory response seen with acute exposure is not muted, a state of prolonged vascular inflammation could be created with potential implications for long-term atherosclerosis development. There is no prior study that we know of that showed pro-inflammatory effects and reduced endothelial function following exposure to medin; however, our findings are complementary to the work by Peng *et al.*[3] showing that vascular smooth muscle cells exposed to medin increased

the production of matrix metalloproteinase-2, a protease that degrades elastin and collagen leading to weakening of the vessel wall. The same group observed that medin was present in the temporal arteries of both elderly patients and patients with giant cell arteritis,[4] with the authors raising the question of the role of medin in arterial inflammation.

4.3 Potential homology with other amyloid proteins

Our results show very similar mechanisms of injury induced on human microvessels by medin when compared to our previous studies on amyloidogenic light chain proteins[6] and β-amyloid,[7,11] despite having widely varying amino acid sequences. The exact basis for this observation remains unknown but other studies suggest a structural homology shared by misfolded proteins. Kayed *et al.*[36] showed that six amyloid proteins with no sequence homology nevertheless showed similar cellular cytotoxicity, and administration of antibody against soluble oligomers culled from various amyloid proteins uniformly protected against cytotoxicity in all six amyloid species. Lashuel *et al.*[37] showed using electron microscopy that three amyloid proteins associated with Parkinson's disease and Alzheimer's disease with widely variant amino acid sequences showed similar annular morphologic tertiary structure conformations that are indistinguishable from each other, which they postulate may underlie the common toxicity among these amyloid proteins. Our group's current and prior findings that medin, amyloid light chains and β-amyloid show similar effects of endothelial dysfunction, oxidative and nitrative stress are consistent with this body of work by other investigators. Another implication of this observation is that the multi-organ toxicity seen with many misfolded proteins may be mediated, at least in part, by common effects on the microcirculation and that therapeutic approaches to mitigate the deleterious effects on the microvasculature may have general, not just protein- or disease-specific, utility.

4.4 Human microvascular model

Our findings also demonstrate similarity in degree and direction of response to medin in peripheral subcutaneous adipose and central leptomeningeal arterioles. This is similar to our results when we exposed living subject adipose, cadaver adipose and leptomeningeal arterioles to Aβ42[11] and when adipose arterioles and atrial coronary arterioles were exposed to AL light chain proteins.[6] This supports the contention that more easily obtainable subcutaneous adipose arterioles could be acceptable surrogates to study pathophysiologic responses in central (cerebrovascular or coronary) arterioles. This has potential translational relevance in offering a new *ex vivo* human model for studying vascular pathophysiology.

4.5 Limitations

This study has several important limitations. Our human *ex vivo* vascular model and endothelial cell experiments only studied acute, and not chronic, effects of medin exposure. It is not known whether chronic exposure will lead to sustained vascular insult and this needs to be established in future studies to better determine the link with human aging-related vascular pathology. The study lacked *in vivo* confirmation and our results should be confirmed *in vivo* with future animal experiments. Despite this limitation, the results of our acute exposure experiments remain salient by providing important foundational knowledge on potential mechanisms of action underlying medin vascular effects that would be relevant starting points in studying *in vivo* pathophysiology and mechanisms. Although it is intuitive to attribute, at least in part, the reduction in endothelial cell proliferation following medin exposure to medin's effect in reducing cell viability based on prior work showing the tight relationship between endothelial cell viability and proliferation,[38] the study did not specifically explore this relationship. Future studies should look into whether medin's effect is also due to dysregulation of known signaling pathways regulating endothelial cell proliferation such as expression of growth factors (e.g. vascular endothelial growth factor) or MAPK signaling.[39] Our results support the possible role of eNOS uncoupling by medin in causing oxidative and nitrative stress, but future studies need to determine if other sources of oxidative stress such as mitochondria or nicotinamide adenine dinucleotide phosphate oxidase, among others, may be affected by medin since a previous study demonstrated multiple sources of superoxide production induced by another type of amyloidogenic protein (light chains in AL).[6] Although we measured only IL-6 and IL-8, in light of activation of NFκB, we anticipate, but did not verify, that other cytokines known to be upregulated by NFκB will also be increased by medin. We limited our study on RAGE and did not formally test whether other endothelial cell receptors known to mediate inflammation, such us toll-like receptors, are involved. The degree of medin's effect on inflammatory cytokine gene and protein expression in endothelial cells is substantial. Our results showed ~27 times increase in IL-8 protein expression (control 1716 ± 398 vs. medin 5 μM 47046 ± 10836 pg/mL, $P < 0.01$, *Figure 6D*). To put this in context, human umbilical veins exposed for 24 h to lipopolysaccharide (100 ng/ml), a classic proinflammatory bacterial agent, increased IL-8 only 4-6 times.[40] Our findings do not explain the discrepancy between the degrees of gene and protein expression increases in IL-8. To investigate this interesting observation, future follow-up studies should be performed to systematically investigate potential causes including mRNA stability, rate of mRNA decay and factors that may be affecting protein expression at the post-transcriptional level. The gender composition of our sources of adipose arterioles is not diversified despite our attempts to actively recruit both male and female participants owing to the current makeup of the elective surgical population in our Veteran's Affairs hospital. Although there is a potential link between medin effects and vascular dysfunction associated with aging, and our results show increased medin aortic content in subjects >55 years old vs. younger, we did not look at aging/senescence markers in our vessels or endothelial cells to further explore these relationships, an area of future study. Although the investigators were careful in using a consistent %weight/volume amount of tissue for loading aortic tissue for western blot, the lack of measurement of a control protein to correct for loading could potentially overestimate or underestimate actual medin tissue content.

4.6 Conclusions

In conclusion, physiologic doses of medin induces RAGE-mediated human peripheral and central arteriole endothelial dysfunction, with dysregulated endothelial cell NO bioavailability, oxidative and nitrative stress leading to reduced endothelial cell viability and proinflammatory signaling via NFκB activation (proposed schema in *Figure 8*). This advances our understanding of the acute effects of medin in vascular function and identifies a potential novel mechanism of vascular injury.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

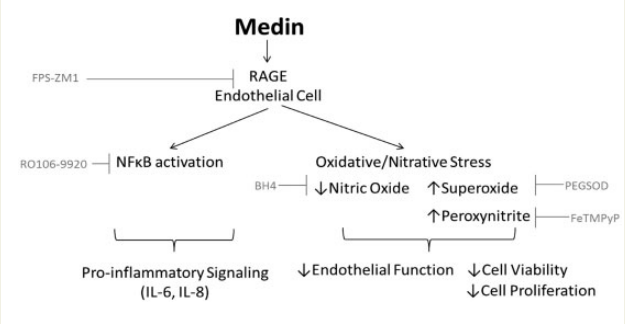

Figure 8 Proposed schema of medin effect on endothelial function. Based on our findings, we propose that medin causes oxidative and nitrative stress in human microvessels leading to endothelial dysfunction and reduced endothelial cell viability and proliferation. Medin also causes pro-inflammatory signaling mediated via activation of NFκB. The effects of medin are mediated via receptor for advanced glycation endproducts.

Acknowledgements

We would like to thank our research volunteers, the surgeons and staff of the Surgery Service of the Phoenix VA, John Hatfield, Camelia Burciu, Peter Reaven, the Carl T. Hayden Medical Research Foundation and the Office of Research of the Phoenix VA. The contents of the work do not represent the views of the Veterans Affairs or the United States government.

Conflict of interest: none declared.

Funding

The study was supported by the Veterans Affairs Merit grant (BX-003767-01, BX007080), National Institutes Health (NIA R21AG044723, NINDS U24NS072026, NIA P30AG19610, NIA RO1AG019795), British Heart Foundation (FS/12/61/29877); the Arizona Department of Health Services (contrast 211002); the Arizona Biomedical Research Commission (4001, 0011, 05-901 and 1001); Michael J. Fox Foundation for Parkinson's Research and Amyloidosis Foundation. The study was based on work supported by the Department of Veterans Affairs and VA employment.

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